CELL WALL LYSIS AND THE RELEASE OF PEPTIDES IN *BACILLUS* SPECIES[1]

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The bacterial cell has a rigid wall which surrounds the cytoplasm enclosed within a membrane. A slimy capsular layer may lie on the outside of the wall but the composition of this material is not considered in the present contribution. Important findings relating to the chemical composition of cell walls have been reported in a number of articles or reviews (1–3) and a brief account of some of them is given. The second part of this contribution presents some of the results obtained from work with spore-bearing organisms of the genus *Bacillus*.

Knowledge of the chemical composition of bacterial cell walls has accumulated over a relatively short time and its present status owes much to Salton and Horne (4) who developed a method, now widely used, for the preparation of essentially homogeneous walls from mechanically disrupted cells. The purification procedure consisted of washing the homogenates with buffer solutions and differential centrifugation. Walls used for chemical analysis were shown to be free from cytoplasmic material by the use of electron microscopy. Cummins and Harris (5) have included treatment of the isolated walls with trypsin and ribonuclease followed by pepsin as additional steps in purification. Under the electron microscope, the separated walls appeared as collapsed, balloon-like structures. The preparations contained lipids, phosphorus, hexosamine, amino acids, and usually carbohydrate but no nucleic acid.

Investigation of cell walls from a few bacterial species showed a marked difference between the composition of those from gram-positive and gram-negative bacteria (2). Walls from gram-negative bacteria contained 10 times more lipid and 10 times less hexosamine than those from gram-positive organisms; the number of amino acids present was greater and included most of those found in proteins, with diaminopimelic acid in addition. Cummins and Harris (5, 6) investigated the cell-wall composition of a large variety of bacteria belonging to different genera and their results allow an important generalization to be made. They used paper chromatography to identify the amino acids, amino sugars, and other sugars present in acid hydrolyzates of walls isolated from gram-positive bacteria and found that only three or four amino acids were present in each wall preparation; these were alanine, glutamic acid, either diaminopimelic acid or lysine, and sometimes also aspartic acid, glycine, or serine. In the case of some cell walls containing lysine and aspartic acid, a cyclic peptide of these two amino acids was found in acid hydrolyzates. This substance is probably not present as such in the cell wall but is formed during acid hydrolysis. A similar cyclic peptide was obtained from bacitracin by Swallow and Abraham (7). Other amino acids were sometimes present in trace amounts. Glucosamine and muramic acid were always present and sometimes galactosamine. The distribution of sugars varied greatly among the species. Cummins (1) suggests that "each bacterial genus may have a distinctive pattern of cell wall constituents, in particular among the amino acids present." Application of this principle may prove useful in the field of bacterial taxonomy. For example, *Corynebacterium pyogenes* gave rise to a mutant which was indistinguishable from *Corynebacterium haemolyticum* and analysis showed glucose to be present in the walls of *C. pyogenes* but absent from walls of the mutant (8). On the basis of cell-wall analyses it was suggested that neither organism is a *Corynebacterium* species and that taxonomically both organisms belong to the genus *Streptococcus*. In the Actinomycetales, cell walls were found to contain sugars, amino sugars, and amino acids (few in number) and thus resembled those of gram-positive bacteria. The mycelial walls of fungi are composed entirely of carbohydrate and therefore the Actinomycetales should not be classified with them but with the bacteria proper (9). Cell wall

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composition of the Actinomycetales has also been investigated by Sohler *et al.* (10) with similar results. However, on the basis of cell-wall composition, *Bacillus sphaericus* would present a problem to the taxonomist. Vegetative cells of three varieties of this species contained no diaminopimelic acid whereas spores contained normal amounts (11). This amino acid was present in vegetative cell walls from all other members of the *Bacillus* group studied.

Certain amino acids found in walls are present as the D-isomers. In cell walls of *Streptococcus faecalis*, 25 per cent of the total alanine and 85 per cent of the total glutamic acid were the D-isomers; walls from a *Lactobacillus* species contained some D-lysine whereas 65 per cent of the total glutamic acid and 54 per cent of the total alanine were the D-isomers (12, 13). Park and Strominger (14) showed that in cell walls of *Staphylococcus aureus* 45 to 50 per cent of the total alanine and 92 to 100 per cent of the total glutamic acid were in the D-form. Salton detected D-alanine in acid hydrolyzates of cell walls by treating developed chromatograms with D-amino acid oxidase followed by 2,4 dinitrophenylhydrazine. The isomer was found to be present in cell walls of a large variety of organisms (15). The most commonly found isomer of diaminopimelic acid in cell walls is *meso* (16). It has been suggested (3) that the function of the D-isomers might be to render the walls resistant to digestion by proteolytic enzymes. In some cases, resistance to attack by such enzymes as trypsin or pepsin could be equally well explained as being due to the absence in cell walls of certain amino acid linkages known to be required for hydrolysis to occur.

According to Work (3), the results of analyses of walls point to the existence in gram-positive bacteria of a common "basal structure" containing the following constituents: a hexosamine component comprising glucosamine and muramic acid and sometimes also galactosamine; a peptide component made up of alanine, glutamic acid, and either diaminopimelic acid or lysine with sometimes also glycine, aspartic acid, or serine; usually a polysaccharide containing not more that four different sugar residues. Other substances may also be attached to the walls, as for example the protein antigens (1). It should be emphasized that diaminopimelic acid and lysine appear to be mutually exclusive.

Weidel and Primosigh (17) have shown that the "basal structure" is present in cell walls of *Escherichia coli*. In this organism the cell wall is composed of two layers and one of these is composed of lipoprotein. Extraction with 90 per cent phenol dissolved the lipoprotein and the remaining layer possessed the chemical composition typical of a gram-positive organism; it supplied rigidity to the whole cell-wall structure. Similar investigation of cell walls from other gram-negative species will probably show that they all have the "basal structure" present.

Soluble nondialyzable components (peptides) with the characteristic composition of the cell-wall "basal structure" have been isolated from exudates of germinating spores (18, 19). An essentially homogeneous component isolated from *Bacillus megaterium* spore exudate had a molecular weight near 15,000 (20). After acid hydrolysis, the following constituents were found: glutamic acid, alanine, and diaminopimelic acid (in the molar ratio of 1:3:1), muramic acid, glucosamine, and acetic acid, with a small quantity of carbohydrate. All the glutamic acid and 25 per cent of the alanine were present as the D-isomers (21). The peptide has been used as a source for isolation studies (22) of muramic acid, a simple method for isolating which in crystalline form is shown in figure 1. The amino sugar was provisionally identified as 3-O-α-carboxyethylhexosamine (23). This structure has been confirmed by synthesis (24) (figure 2), the infrared spectra of the natural and

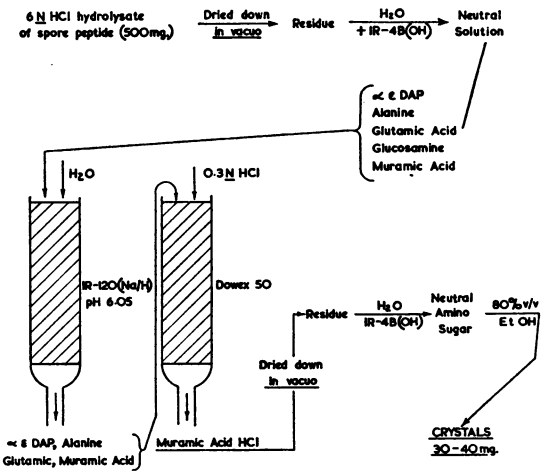

Figure 1. Isolation of muramic acid from *Bacillus megaterium* spore peptide.

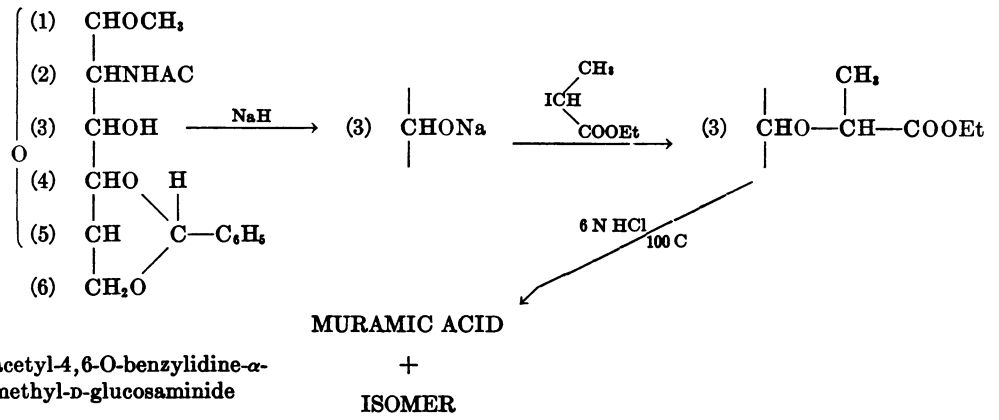

Figure 2. Synthesis of muramic acid (24)

synthetic substances being identical. Muramic acid possesses carboxyl and reducing groups and in the cell wall, where it is present as the N-acetylated derivative, it possibly links an amino acid via a peptide linkage (14) to hexosamine via a glycosidic linkage.

Muramic acid reacts as a hexosamine when the usual methods of detecting or determining amino sugars are used. It can be distinguished from other naturally occurring hexosamines in the following ways: (a) in the Elson and Morgan reaction or modifications of it, muramic acid gives a colored derivative with an absorption maximum at 510 to 520 mμ (19, 22), whereas derivatives of glucosamine or galactosamine have absorption maxima near 530 mμ; (b) on treatment with ninhydrin, followed by chromatography, the product from muramic acid reacts as a pentose but has an R_f value different from that of ribose, xylose, lyxose, or arabinose (22); (c) in Barker and Summerson's test for lactic acid, muramic acid gives a positive reaction (23). It is easily separated from glucosamine or galactosamine by means of the ion-exchange resin IR-120 buffered to pH 6.05 (22). Muramic acid is not absorbed by the resin whereas the other two amino sugars are.

The significance of the presence of peptide in spore exudates was not understood and Cummins (1) suggested that it could be a "soluble fraction of the cell wall elaborated by the germinating protoplasm." This seemed unlikely since similar peptides were present in extracts of mechanically disintegrated resting spores (19). Coats were isolated from disintegrated spores and their chemical composition was investigated (25). In the case of *B. megaterium*, the preparations contained relatively large amounts of the peptide constituents and these were released as a nondialyzable component on incubation of the coats suspended in buffer solutions. Spore coats of *Bacillus cereus* contained little peptide but spore extracts and exudates contained relatively large amounts. Peptide release from the coats could have been due to the activity of an enzyme system which was activated during germination or mechanical disintegration of spores. Results obtained during an investigation of the biochemical changes occurring during sporulation in *Bacillus* species (26) gave support to this hypothesis. *B. cereus* was grown in potato-extract medium and cells at various stages of growth and sporulation were harvested. After washing, the bacteria were disintegrated and separated into soluble and insoluble fractions. The results of analysis of the fractions for diaminopimelic acid and amino sugars showed that in nonsporulating cells, these constituents were associated with the insoluble fraction, whereas in sporulating cells and spores they appeared in the soluble fraction (table 1). Also, an extract of sporulating cells released material containing diaminopimelic acid and amino sugars from the insoluble fraction of nonsporulating cells. The insoluble fractions consisted essentially of cell walls and it appeared that a cell-wall lytic enzyme was present in sporulating cells and spores. Extracts of apparently clean resting spores of this organism and of *Bacillus anthracis* contained lytic enzyme, which was partially purified (27). With a substrate of cell walls, previously heated at 100 C for 15 min to

TABLE 1

Change in distribution of diaminopimelic acid between the insoluble and soluble fraction of Bacillus cereus cells during sporulation (26)

Age and Appearance of Cultures	Whole Cells		Insoluble Fraction		Soluble Fraction	
	Hexosamine; g/100 g dry wt	DAP*	Hexosamine; g/100 g dry wt	DAP	Hexosamine; g/100 g dry wt	DAP
6 hr, vegetative	13	+	38	++	3.7	−
12 hr, vegetative	12	+	37	++	2.2	−
14 hr, first signs of sporulation	13	+	35	++	4.4	−
16 hr, sporulating	13	+	4.8	Trace	8.4	+
18 hr, sporulation almost complete	13	+	3.2	Trace	12	+
36 hr, free spores	8.1	+	1.8	−	9.2	+

* Diaminopimelic acid; +, 1 to 2 per cent; ++, 2 to 4 per cent.

prevent spontaneous lysis, optimal lytic activity occurred near 57 C and pH 7.5 and was stimulated by the presence of Co^{++}, Mn^{++}, Cu^{++} and Ni^{++}. The main product of lysis was a nondialyzable component containing the characteristic constituents of the "basal structure." The lytic enzyme also attacked cell walls from other *Bacillus* species. In order to show that the function of the enzyme in the germinating spore was to release peptide from the coat it was necessary to obtain spore-coat preparations which contained significant amounts of the enzyme substrate. When coats were isolated from spores of *B. cereus* which had been disintegrated in buffer, pH 9.6, they were found to contain nearly three times the hexosamine present in coats from spores disintegrated at pH 7.0. The hexosamine-rich spore coats, previously heated at 100 C for 15 min to prevent spontaneous release of peptide, were incubated with an extract from spores disintegrated at pH 7.0. Peptide was released which established that the coats contained substrate for the lytic enzyme present in spores. Peptide was also released from spore coats of *B. megaterium* by the action of the enzyme from *B. cereus* spores. The lytic enzyme did not attack intact resting spores.

The spore develops in the vegetative cell, which thus becomes a sporangium. It is by no means certain what happens to the vegetative cell wall when the spore is released. In *Clostridium* species it appears that at least part of this structure is retained as an outer membrane around the spore. It is the opinion of some workers that the wall of the sporulating cell forms the exosporium which exists as an outer coat around spores of several *Bacillus* species. Spores of several varieties of *B. cereus* had exosporia whereas these structures appeared to be absent from spores of *B. megaterium* and *B. subtilis*. It seems, however, that in *Bacillus* species at least, the greater part of the vegetative cell wall is dissolved away before the developed spore is released. If this is true, then soluble components containing the characteristic constituents should appear in the medium during spore release. Culture filtrates from *B. cereus* organisms at various stages of growth and sporulation were hydrolyzed and the hydrolyzates analyzed for amino sugars and diaminopimelic acid (28). Results showed that a large increase in the concentration of these substances in the culture filtrate occurred during spore release (table 2); they were found to be present in a nondialyzable peptide of the characteristic type. It was concluded that at least part of the sporangial wall was dissolved away to allow release of the spore. It appears likely that the exosporium of *B. cereus* does not have a composition similar to that of the vegetative cell wall, from the results obtained by Dr. J. R. Norris of Leeds University (*personal communication*). He treated spores with a highly active preparation of lytic enzyme from *B. cereus* spores and examined the effect by means of electron microscopy. No evidence of lysis of the exosporium was obtained.

It was not known whether the enzyme present in spores, or another enzyme, was responsible for lysis of the sporangial wall during spore release. When thick suspensions of washed sporulating cells of *B. cereus* were incubated in buffer

TABLE 2

Diaminopimelic acid (DAP) and amino sugar contents of acid hydrolyzed culture medium-filtrates from vegetative and sporulating cells of Bacillus cereus (28)

Incubation Period of Culture	State of Cells	Dry Wt Cells/ml	Culture Medium Filtrates	
			DAP	Amino sugars (as glucosamine)
hr		*mg*	*μg/ml*	*μg/ml*
10.5	Vegetative forms	1.37	1.8	20.0
17.75	Intracellular spores	1.40	3.0	24.0
18.75	Intracellular spores	1.40	4.4	32.0
22.0	Spores	1.30	9.0	85.0

at 37 C, a partial autolysis occurred, resulting in the freeing of mature and immature spores (28). The autolysate contained lytic enzymes which attacked vegetative cells and cell-wall preparations, releasing peptides of the characteristic constitution. Two water-soluble lytic systems, with pH optima near pH 4.5 and 8.0, respectively, were separated from the autolysate. Activity of the enzyme with a pH optimum of pH 4.5 was unaffected by the presence of Co^{++} whereas the activity of the other enzyme was progressively stimulated in the presence of increasing amounts of this ion (0 to 100 μg/ml). It is probable that the enzyme most active at pH 8.0 in the presence of Co^{++} is identical to the one found in extracts of disintegrated spores. No evidence was found for the presence of the other lytic enzyme in spores of *B. cereus*. The presence of highly active cell-wall lytic enzymes in sporulating cells and the appearance of soluble cell-wall components in the medium during spore release suggest that the vegetative cell wall as such does not form the exosporium of *B. cereus* spores. Using suspensions of highly purified vegetative walls from nonsporulating cells as substrate, enzyme preparations produced a 95 per cent drop in turbidity.

Lytic enzymes with similar activity have been obtained from sporulating cells of other *Bacillus* species. Their action on cell walls and on heat-killed, intact vegetative cells resembles that of lysozyme. Lysozyme attacks cell walls specifically (29) and it also attacks certain mucopolysaccharides of animal origin. Protoplasts of various bacteria have been obtained by treating suspensions of viable cells in sucrose solutions of appropriate osmotic pressure with lysozyme (30). Relatively stable suspensions of protoplasts of *B. megaterium* and *B. cereus* were obtained when suspensions of viable organisms in sucrose solutions at pH 7.5 were incubated with enzyme preparations from sporulating cells of these organisms (31).

Peptides from spores and vegetative cell walls of *B. megaterium* were attacked by lysozyme; digestion mixtures gave positive results for N-acetylhexosamine and reducing sugars (25, 27). Spore peptide was broken down into approximately equal proportions of dialyzable and nondialyzable fractions, both of which contained all the basal cell-wall constituents; a dimer of hexosamine and muramic acid found by Salton (32) in lysozyme digests of whole cell walls was also present in spore-peptide digests. Greater degradation of spore or vegetative cell-wall peptides occurred in the presence of an extract of the gut of *Helix aspersa* which contains β-glucosaminidase (33). Thus it is evident that the cell-wall lytic enzymes present in sporulating cells or spores differ from lysozyme and β-glucosaminidase in their mode of action.

The lytic enzymes of sporulating *Bacillus* species appear to be involved in the sporulation cycle; as a result of their activity, soluble peptide material is released from the sporangial wall and from the spore coat. Other lytic enzymes associated with *Bacillus* species have been described (34–36). An autolytic substance obtained from sporulating cultures of *B. cereus* var. *terminalis* lysed whole vegetative cells of this organism (34) but the enzyme substrate was not defined. The activity of a lytic enzyme demonstrated in autolysates of *B. subtilis* vegetative cells (35) resembled that of the enzymes described, attacking vegetative-cell wall preparations of this organism; this system appeared in nonsporulating cultures. A bacteriolytic principle associated with nonsporulating cells of *B. cereus* (36) attacked the protoplast of this organism, leaving the cell wall apparently unchanged.

In this contribution, attention has been focused on the "basal structure" of cell walls and peptides of similar composition found in spores. Other important components of cell walls have not been considered. Work (3) suggests that "the general inference to be drawn on

over-all structure of bacterial cell walls is that they consist of an insoluble groundwork of chains of acetylated glucosamine and muramic acid bound in some unspecified way to peptides; polysaccharide units and phosphorylated compounds may also be attached." An example of another unit attached to the basal structure is the polysaccharide of *B. anthracis* (37, 38) which contains equivalent amounts of galactose and acetylglucosamine with a small peptide moiety. It was found that the peptide moiety contained diaminopimelic acid and muramic acid (39), suggesting that it was composed of cell-wall peptide.

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